The Antiapoptotic Actions of Mood Stabilizers

Molecular Mechanisms and Therapeutic Potentials

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ABSTRACT: Two primary drugs used to treat bipolar mood disorder are lithium and valproate. Emerging evidence supports the notion that both mood stabilizers have neuroprotective effects. In primary cultures of rat cerebellar granule cells and cortical neurons, lithium and valproate robustly and potently protect against glutamate-induced, *N*-methyl-D-aspartate (NMDA) receptor–mediated excitotoxicity. The neuroprotective mechanisms involve inactivation of NMDA receptors through inhibition of NR2B tyrosine phosphorylation, activation of cell survival factors such as the PI 3-kinase/Akt signaling pathway, and induction of neurotrophic/neuroprotective proteins, including brain-derived neurotrophic factor, heat-shock protein (HSP), and Bcl-2. Both drugs are also effective against other forms of insults such as ER stress in neurally related cell types. The molecular targets likely involve glycogen synthase kinase-3 (GSK-3) and histone deacetylase (HDAC) for lithium and valproate, respectively. In a rat cerebral artery occlusion model of stroke, postinsult treatment with lithium or valproate reduces ischemia-induced brain infarction, caspase-3 activation, and neurological deficits, and these neuroprotective effects are associated with HSP70 upregulation and, in the case of valproate, HDAC inhibition. In a rat excitotoxic model of Huntington's disease in which an excitotoxin is infused into the striatum to activate NMDA receptors, short-term lithium pretreatment is sufficient to protect against DNA damage, caspase activation, and apoptosis of striatal neurons, and this neuroprotection is concurrent with Bcl-2 induction. Moreover, lithium treatment increases cell proliferation near the site of striatal injury, and some newborn cells have phenotypes of neurons and astroglia. Thus, lithium and valproate are potential drugs for treating some forms of neurodegenerative diseases.

KEYWORDS: BDNF; Bcl-2; excitotoxicity; heat-shock protein; lithium; neurodegeneration; neurogenesis; neuroprotection; valproate

Lithium and valproate are two of the most prominent drugs approved by the United States Federal Food and Drug Administration (FDA) used in the treatment of bipolar mood disorder. This mental illness, which includes symptomatology of alternating depression and mania, is recognized by the World Health Organization as a leading debilitating neuropsychiatric disorder that affects about 1.3% of both sexes globally.[1]

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Over 50 years ago, lithium was discovered to be efficacious in the control of acute mania. Additional findings showed it to be prophylactic against recurrent manic and depressive episodes, and to be augmentative of the activity of classical antidepressants in some depressive patients. Recent evidence has supported that certain anticonvulsants, for example, valproate, carbamazepine, and lamotrigine, also have clinical efficacy in the treatment of bipolar disorder (for review, see refs. 2 and 3). Valproate is effective, like lithium, in preventing manic episodes; however, as with lithium, it is also less beneficial in the prevention of depression. Research continues to focus on the underlying mechanisms of lithium and valproate, which still remain uncharacterized, and current studies have led to an appreciation of the complexity of bipolar disorder etiology and that the effects of mood stabilizing drugs may result from multiple routes of action (for review, see ref. 4). Since mood stabilizers require chronic treatment for their beneficial effects, it has been hypothesized that alterations of signaling pathways and gene expression may be involved. Hence, great interest has been raised in the investigation of lithium and other mood stabilizing agents on gene expression and cellular signaling in both basic and preclinical laboratories.

Our pioneering studies have shown that chronic exposure of cultured rat cerebellar granule cells with therapeutic concentrations of lithium increases m_3-muscarinic acetylcholine receptor–mediated second messenger production as well as c-fos and m_3-receptor expression.[5] Lithium at therapeutic doses increases the activities of two prominent transcription factors, AP-1 and CREB (cyclic AMP–response element binding protein), in cultured cerebellar granule cells and in distinct brain areas (e.g., frontal cortex, amygdala, hippocampus, and cerebellum) in rats treated by diet for 2 or 4 weeks.[6] A growing body of evidence suggests that m_3-muscarinic receptors and the DNA binding activities of AP-1 and CREB have prominent roles in the regulation of cell viability. Accordingly, we proposed that mood stabilizers have neuroprotective and neurotrophic actions.

Glutamate-induced excitotoxicity in discrete brain areas has been linked to a variety of neurodegenerative diseases such as stroke, Huntington's disease, ALS, brain trauma, cerebellar degeneration, spinal cord injury, and possibly Alzheimer's disease and Parkinson's disease (for review, see refs. 7–9). Therefore, we investigated the effects of lithium on glutamate-induced excitotoxicity using primary cultures of cerebellar granule cells (CGCs) as a model. We found that chronic lithium treatment robustly reduces glutamate-induced excitotoxicity and this can be completely blocked by *N*-methyl-D-aspartate (NMDA) receptor antagonists.[10] This neuroprotection is long-lasting, occurs at therapeutically relevant concentrations ($EC_{50} \approx 1$ mM), and requires 6–7 days of pretreatment for maximal effects. The action is also unrelated to lithium's inhibition of inositol monophosphatases in that coaddition with excessive myoinositol fails to reverse lithium's neuroprotective effects. The neuroprotection by lithium involves blockade of the apoptotic component of glutamate excitotoxicity and is due, in part, to a reduction in NMDA receptor–mediated intracellular calcium increase. Because long-term pretreatment is necessary to elicit these neuroprotective effects, gene expression is likely involved. In support of this notion, we found that treatment with lithium induces the cytoprotective Bcl-2 protein, but downregulates the proapoptotic proteins such as p53 and Bax.[11] Conversely, glutamate treatment downregulates Bcl-2, but upregulates p53 and Bax, and these actions of glutamate are reversed by lithium pretreatment. It has also been reported that chronic lithium and VPA treatments upregulate Bcl-2 in the brain of rats.[12]

Lithium also exerts its effects on the cell survival factor, Akt, which is a downstream kinase of phosphatidylinositol 3-kinase (PI 3-kinase). Lithium treatment of rat CGCs was found to rapidly activate PI 3-kinase, resulting in increased phosphorylation of Akt at Ser473 and enhanced Akt activity.[13] Additionally, induced Akt activation by lithium is associated with enhanced phosphorylation levels at Ser21 of glycogen synthase kinase-3α (GSK-3α), previously thought to be directly inhibited by lithium[14] and a known phosphorylation substrate of Akt. The presence of a PI 3-kinase inhibitor, LY 294002, abolishes lithium-induced phosphorylation of GSK-3α and GSK-3β, further suggesting the involvement of the PI 3-kinase/Akt signaling pathway. In contrast, glutamate treatment causes a rapid, but reversible loss of Akt (Ser473) phosphorylation and activity apparently through protein phosphatase activation,[13] and these effects are also prevented by lithium.

CREB is a downstream signaling target of the mitogen-activated protein kinase (MAP kinase) system that, upon phosphorylation, has a major role in mediating adaptive responses at glutamatergic synapses and cell survival by promoting the expression of cell-protective proteins such as BDNF and Bcl-2 (for review, see ref. 15). In CGCs, we showed that toxic concentrations (≥50 μM) of glutamate induce an NMDA receptor–dependent decrease in CREB phosphorylation at Ser133 (and hence CREB inactivation),[16] and that chronic lithium treatment, concurrent with the neuroprotective effects, suppresses glutamate-induced loss of phosphorylated CREB (p-CREB). Studies using selective inhibitors demonstrate that protein phosphatase 1 predominantly regulates glutamate-induced decrease in p-CREB levels. The MEK/ERK MAP kinases are upstream of CREB and known to be involved in cytoprotection (for review, see ref. 15). Treatment with glutamate induces a rapid increase in MEK activity in CGCs and this is potentiated by long-term lithium pretreatment, an effect blocked by the MEK inhibitor, PD 98059.[16] Our results suggest that long-term lithium exposure is necessary to maintain higher activated CREB levels in CGCs subjected to glutamate excitotoxicity and that this may be the result of the inhibition of protein phosphatase 1 as well as promotion of the activities of MEK/ERK by itself.

c-Jun N-terminal kinase (JNK) and p38 kinase, another member of the MAP kinase family, are activated by site-specific phosphorylation in response to a variety of apoptotic insults (for review, see ref. 17). The p38 kinase and JNK often act synergistically to enhance AP-1 binding activity. Using rat CGC cultures, we tested the hypothesis that AP-1 binding activation is involved in glutamate excitotoxicity and is a target of lithium as part of lithium-induced neuroprotection. We found that glutamate, through NMDA receptor activation, causes a rapid activation of JNK and p38 kinase, which then phosphorylate c-Jun (at Ser63) and p53 (at Ser15), leading to a robust increase in AP-1 binding that precedes apoptotic death.[18] Long-term lithium pretreatment can block these glutamate-induced apoptotic effects; moreover, SB 203580, a p38 kinase inhibitor, selectively prevents glutamate-induced p38 activation, p53 phosphorylation, and AP-1 binding activation, and shows neuroprotection. Curcumin, a potent inhibitor of AP-1 activity, inhibits glutamate-induced AP-1 binding and protects CGCs from glutamate excitotoxicity. Our results suggest that NMDA receptor–mediated apoptotic death requires both the actions of JNK and p38 to enhance AP-1 binding and p53 phosphorylation (and hence p53 stabilization). Moreover, suppression of receptor-mediated activation of the JNK/p38 MAP kinase pathway and subsequent AP-1 activation play a role in lithium neuroprotection.

CGCs have also been used to test the neuroprotective effects of another mood stabilizer, valproate, against excitotoxicity. Treatment of CGCs with SYM-2081 [(2*S*,4*R*)-4-methylglutamate], an inhibitor of excitatory amino-acid transporters and an agonist of low-affinity kainate receptors, induces apoptotic cell death.[19] The SYM-2081-induced excitotoxicity is blocked by an NMDA receptor antagonist and associated with a rapid and robust nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene previously shown to be proapoptotic (for review, see ref. 20). Recent studies identified histone deacetylase (HDAC) as a direct target of valproate.[21,22] We found that pretreatment of CGCs with valproate or other HDAC inhibitors such as butyrate and trichostatin A induces a time- and concentration-dependent neuroprotection against SYM-2081 excitotoxicity.[19] Moreover, the valproate neuroprotection is associated with suppression of SYM-2081-induced nuclear accumulation of GAPDH, which is associated with hyperacetylated histone H3, including Lys9-acetylated histone.

Primary cultures of rat cerebral cortical neurons have also been used in our studies because abnormalities in volume, cell density, and neuronal size have been found in the cerebral cortex of bipolar patients (for review, see refs. 23 and 24). Rat cortical neurons are highly vulnerable to glutamate excitotoxicity, with an LD_{50} of only 8–10 μM. This form of toxicity is also NMDA receptor–mediated in this cell type.[25] Protracted lithium pretreatment for 5–6 days is necessary for complete protection against glutamate excitotoxicity, and significantly less lithium is required compared to CGC cultures.[25] Thus, neuroprotective effects of lithium are observed in the subtherapeutic and therapeutic concentrations of 0.2–1.6 mM, with an optimal dose at 1 mM. Additionally, the neuroprotection is specific for lithium since other monovalent ions (Rb and Cs) and classical antidepressants (imipramine, desipramine, clomipramine, and fluoxetine) are ineffective.

In cultured cortical neurons, lithium neuroprotection is also associated with an attenuation of NMDA receptor–mediated calcium influx.[25] Concomitant with this inhibition, lithium induces a time-dependent selective decrease in phosphorylation of Tyr1472 of the NR2B receptor subtype. Mutation studies have shown that phosphorylation of NR2B at Tyr1472 is crucial in mediating NMDA receptor channel activity.[26] Thus, lithium-induced decrease of NR2B Tyr1472 phosphorylation likely plays a role in the observed reduction of NMDA receptor–mediated Ca^{2+} influx. Tyrosine phosphorylation of NR2B (and NR2A) is mediated by the Src family kinases, which bind to scaffolding proteins in the NMDA receptor complex. Levels of phosphorylated Src kinase at Tyr416, an index of Src activation, were found to be reduced after long-term treatment with LiCl (1 mM).[27] Moreover, an Src kinase inhibitor, SU-6656, and an NR2B antagonist, ifenprodil, partially block glutamate excitotoxicity. Together, these data are suggestive that the inactivation of Src kinase and resulting NR2B NMDA receptor inhibition is a mechanism by which lithium confers neuroprotection against glutamate toxicity.

We next studied the neuroprotective roles of BDNF, a neurotrophin that is essential for neuronal survival, synaptic plasticity, and cortical development, and likely plays a role in the actions of antidepressants (for review, see refs. 15 and 24). We hypothesized a critical role of BDNF/TrkB in the mediation of lithium neuroprotection against glutamate excitotoxicity in rat cortical neurons and diverse signaling pathways mediated by mood stabilizers.[28] Results showed that a Trk tyrosine kinase inhibitor, K252a, and a BDNF-neutralizing antibody can suppress lithium-induced

neuroprotection against excitotoxicity, which suggests the involvement of BDNF/TrkB activation. Evidence supporting this hypothesis comes from our findings in cortical neurons that lithium transiently increases intracellular BDNF followed by an increase in levels of phosphorylated TrkB (at Tyr490). Thus, long-term lithium enhances BDNF expression, secretion, and subsequent activation of TrkB receptors. In addition, we found in cortical neurons that exon III mRNA levels of BDNF and promoter III activity of the BDNF gene are increased by lithium treatment.[29] It is important to note that lithium does not protect cortical neurons derived from either heterozygous (+/–) or homozygous (–/–) BDNF knockout mice from glutamate excitotoxic death, while it completely protects cultures derived from wild-type mice littermates.[28] Altogether, our results suggest that in cortical neurons lithium causes elevation of BDNF to a critical level, which is essential for this drug to exert its protective effects against excitotoxicity, and that the BDNF/TrkB signal pathway likely mediates many of the reported downstream effectors. It is also noteworthy that valproate similarly protects against glutamate excitotoxicity in cortical neuronal cultures[25] and substantially increases neuronal culture life spans.[30] The latter protective effect is mimicked by HDAC inhibitors and associated with histone hyperacetylation and induction of heat-shock protein 70.[30]

In addition to glutamate excitotoxicity, lithium can also protect against other forms of insults in CNS neurons and neurally related cell lines. Our studies revealed that lithium rescues rat CGC cultures from apoptosis induced by potassium deprivation, supratherapeutic concentrations of anticonvulsants (phenytoin and carbamazepine), and spontaneous death.[31] Lithium also protects pheochromocytoma PC 12 cells from apoptosis induced by thapsigargin, an inhibitor of Ca^{2+}-ATPase on the endoplasmic reticulum (ER) and an inducer of Ca^{2+} release from the ER.[32] This protection is concomitant with suppression of thapsigargin-triggered intracellular calcium release from the ER, and induction of Bcl-2 and Grp 78 (glucose-regulated protein 78). The protection against ER stress in PC 12 cells is also induced by valproate pretreatment.[32] Moreover, treatment of PC 12 cells or CGCs with beta-amyloid peptide (1–42) for 24 h induces a decrease in cellular redox activity, which is accompanied by cell death, and this effect is markedly attenuated by long-term (7 days) lithium pretreatment.[33] The neuroprotective effects in beta-amyloid-treated cells are temporally correlated with upregulation of Bcl-2 protein levels. Accordingly, we have proposed that lithium's use in the treatment of Alzheimer's disease should be reexamined.[33] This notion is strengthened by the recent report that inhibition of GSK-3 by lithium results in the suppression of beta-amyloid production from its precursor protein.[34] Indeed, mounting evidence from various laboratories further supports the view that lithium can be neuroprotective against diverse forms of death insults, suggesting that it is a multifunctional neuroprotectant (for review, see refs. 24 and 35–38).

Based on the findings that both lithium and valproate are protective against glutamate excitotoxicity and other insults in CNS neurons, we hypothesized that these two mood stabilizers may have beneficial effects in multiple forms of neurodegenerative diseases, particularly those linked to excitotoxicity. Stroke is one neurodegenerative disease with a glutamate excitotoxicity component, and is a major cause of mortality and morbidity worldwide. Following brain ischemia, a substantial excess release of glutamate occurs and the resulting overstimulation of glutamate receptors leads to brain damage. With this as background, we investigated the effects of mood stabilizing drugs in a rodent model of stroke.

Our pilot studies employed a permanent middle cerebral artery occlusion (MCAO) stroke model in rats in which a nylon suture was inserted via the left external carotid artery into the left internal carotid artery to occlude the origin of the left middle cerebral artery, which results within 24 h in a massive brain infarct of the middle cerebral artery territory, including the cerebral cortex and the lateral segment of the caudate nucleus.[39] By pretreating rats for 16 days with subcutaneous injections of LiCl at therapeutic concentrations, we observed a >50% reduction in infarct volume. Moreover, the MCAO-induced neurological deficits such as hemiplegia and posture abnormality were also significantly suppressed.[39] Furthermore, our laboratory showed that lithium treatment markedly increases the number and intensity of neurons expressing Bcl-2 in brain areas vulnerable to ischemic insult.[40,41] As best as can be determined, this is the first demonstration of lithium protection against brain injury in an animal model of stroke, and these neuroprotective effects of long-term lithium treatment have been confirmed by others.[42]

The next rational step was to investigate whether postinsult treatment with lithium is neuroprotective in the MCAO model, which would support the use of this mood stabilizer as a potential therapy for acute stroke. We used a transient ischemia model in which rats were subjected to MCAO for 1 h followed by reperfusion, a model likely more related to the pathophysiology of acute stroke in humans. LiCl (0.5–3.0 mEq/kg) administered by subcutaneous injection immediately after MCAO was found to reduce infarct volume measured 24 h later.[43] The effective dose range used maintains plasma lithium concentrations at the therapeutic to subtherapeutic level at 12 and 24 h after injection. Similar drug treatments administered every 24 h cause a dose-dependently reduced neurological deficit score as measured by motor, sensory, and reflex tests over a period of 1 week or longer. Infarct volume and neurological deficit reductions were observed at therapeutic doses (0.5 and 1.0 mEq/kg) of lithium and even when injected (1.0 mEq/kg) at least up to 3 h after MCAO induction.[43] Suppression of MCAO/reperfusion-induced caspase-3 activation and DNA damage in neurons additionally supports the use of lithium as a neuroprotective agent.

Heat-shock protein 70 (HSP70) is known to play a major cytoprotective part in apoptosis and is induced in the ischemic penumbra where neuronal recovery is predominantly found (for review, see ref. 43). Moreover, transgenic mice overexpressing rat HSP70 showed reduced brain infarction in a permanent focal ischemia model.[44] We therefore investigated the role that heat-shock response may play in lithium neuroprotection, especially in light of a recent report that lithium inhibition of GSK-3 is associated with activation of heat-shock factor-1 (HSF-1),[45] a transcription factor for HSP70. Immunohistochemistry and Western blotting showed that lithium time-dependently superinduces HSP70 in the ischemic brain.[43] Moreover, EMSA showed that this HSP70 induction is preceded by a marked increase in the DNA binding activity of HSF-1 to the heat-shock element. Collectively, our research has demonstrated that postinsult lithium administration robustly protects neurons in the rat MCAO/reperfusion stroke model and that this lithium treatment may upregulate heat-shock response as part of the neuroprotective mechanisms.

Recently, we investigated the neuroprotective efficacy of valproate using the same MCAO/reperfusion paradigm in rats. Valproate (300 mg/kg) injected subcutaneously after ischemic induction, followed by injections every 12 h, significantly reduces infarct size and neurological deficits observed 48 h later,[46] and also markedly suppresses MCAO-induced caspase-3 activation in the cortex. Additionally, VPA increases

acetylated histone H3 levels in a time-dependent manner in cortex and striatum of both ipsilateral and contralateral hemispheres, suggesting that HDAC activity is inhibited *in vivo*. Furthermore, valproate neuroprotection is associated with a robust increase in HSP70 levels in both ipsilateral and contralateral cortical and striatal areas.[46] Thus, similar to the *in vitro* results, valproate-induced HSP70 upregulation could be triggered by HDAC inhibition and participates in the neuroprotection against cerebral ischemia.

Huntington's disease (HD) is a devastating neurodegenerative disorder involving a selective loss of neurons in the brain, notably in the striatum and, to a lesser extent, in the cortex, and is characterized by involuntary hyperkinetic movement, impaired cognition, and psychiatric syndromes, especially depression.[8,47] It is widely believed that an expansion of CAG repeats in the exon I region of the gene encoding huntingtin[48] is the mutation responsible for HD; however, the direct link between the mutation and neurodegeneration found clinically remains obscure. In the investigation of HD, a frequently used animal model utilizes the infusion of quinolinic acid (QA), as a neuronal excitotoxin, into the striatum (for review, see ref. 49). Striatal QA infusion causes the death of medium-sized spiny neurons via activation of NMDA receptors and produces many of the neuroanatomical changes found in HD. In initial experiments, we pretreated rats with subcutaneous injections of therapeutic doses of LiCl for 16 days prior to unilateral infusion with QA. Results showed that lithium pretreatment markedly reduces the sizes of QA-induced striatal lesions,[40] and detailed analysis showed the number and intensity of DNA-damaged striatal neurons near the QA infusion site to be markedly decreased when compared to controls. Moreover, an increase in Bcl-2 immunostaining in medium-sized projection neurons and large interneurons in the striatum as well as in the frontal cortex is correlated with long-term lithium-induced neuroprotection. These early QA excitotoxicity HD studies were expanded by investigating the neuroprotective effects of short-term lithium pretreatment, characterizing specific striatal cell populations that could be protected, and investigating possible underlying mechanisms. Our results showed that subcutaneous LiCl injections given 24 h before and 1 h after unilateral infusion of QA into the striatum reduce the number of neurons showing caspase-3 activation and DNA damage, and decrease the loss of neurons immunostained by NeuN (a neuronal marker). However, this acute treatment fails to prevent the loss of NADPH-diaphorase-positive striatal interneurons.[41] Additionally, 24-h lithium pretreatment also upregulates Bcl-2 protein levels in the striatal tissue and increases the number and density of Bcl-2-immunostained striatal neurons.

Because lithium has the ability to increase neurogenesis in the rat hippocampus *in vivo*[50] and to stimulate neuroblast proliferation in rat neuronal cultures,[51] we investigated the effects of lithium on striatal cell proliferation in the rat QA model. One week after QA infusion of rats not treated with lithium, we found very few BrdU-labeled (or replicating) cells in the injured striatum near the QA injection site,[41] but densely packed BrdU-positive cells located in the subventricular zone (SVZ) of both ipsilateral (QA-infused) and contralateral (intact) striata. Of interest, in rats pretreated with lithium, we found a large number of BrdU-labeled cells near the QA-infused site, and this increase in the injured striatum is accompanied by a reduction in BrdU-labeled cells in the SVZ. Moreover, although the majority of proliferating cells were found to be neither mature neurons nor astrocytes, a small population of NeuN-stained cells with strong BrdU-labeling and some GFAP-

expressing cells with weak labeling were observed. Thus, it appears that lithium can induce the migration of progenitor cells from the SVZ to the area damaged by QA infusion and/or can stimulate localized proliferation of neuronal and astroglial progenitor cells in the injured striatum. Both this cell-proliferating effect of lithium as well as its antiapoptotic property may underlie the neuroprotection observed in the QA-induced excitotoxicity model.

In conclusion, accumulating evidence strongly supports that the mood stabilizers, lithium and valproate, exert multiple effects that lead to neuroprotection against glutamate excitotoxicity and other insults in cultured CNS neurons and neurally related cell types. In rodent models of stroke, postinsult treatments with lithium and valproate reduce infarct volume and improve functional outcome. In the QA model of HD, both short- and long-term lithium pretreatments reduce the excitotoxin-induced loss of striatal spiny neurons. *In vitro* studies show that induction of BDNF is a prerequisite for inducing the multiple actions and resultant protective effects. Lithium also has the ability to enhance cell proliferation in neuronal cultures as well as in the vicinity of a brain injury, and this particular action may contribute to its observed neuroprotective effect. In light of the recent evidence showing the loss of neurons and glial cells in discrete brain areas of unipolar and bipolar patients and the possible involvement of glutamate in stress-induced depression, the neuroprotective effects of lithium may be the mechanisms that give clinical relevance to the use of this drug in the treatment of bipolar patients. Moreover, in view of the role that glutamate excitotoxicity plays in the pathophysiology of several neurodegenerative diseases, lithium, valproate, and perhaps other mood stabilizers may be found to have an expanded use in the suppression or prevention of neurodegeneration, in addition to their mainstay use to treat bipolar disorder.

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